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Short Communication

IL-7 and immobilized Kit-ligand stimulate serum- and stromal cell-free cultures of precursor B-cell lines and clones

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Long-term proliferating, D_HJ_H-rearranged mouse precursor B-cell lines have previously been established in serum- and IL-7-containing media from fetal liver, but not from bone marrow. Serum and stromal cells expose these pre-B cells to undefined factors, hampering accurate analyses of ligand-dependent signaling, which controls pre-B cell proliferation, survival, residence and migration. Here, we describe a novel serum-free, stromal cell-free culture system, which allows us to establish and maintain pre-B cells not only from fetal liver, but also from bone marrow with practically identical efficiencies in proliferation, cloning and differentiation. Surprisingly, recombinant kit-ligand, also called stem cell factor, produced as a kit-ligand-Fc fusion protein, suffices to replace stromal cells and serum, provided that it is presented to cultured pre-B cells in an optimal density in plate-bound, insolubilized, potentially crosslinking form. Additional recombinant CXCL12 and fibronectin have a minor influence on the establishment and maintenance of pre-B cell lines and clones from fetal liver, but are necessary to establish such cell lines from bone marrow.

Keywords: IL-7 · Kit-ligand · Pre-B cell · Proliferation · Serum-free · Stem cell factor · Stromal cell-free

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Introduction

Hematopoietic stem cells (HSCs) and their progeny on the way to B-lymphocytes—multipotent progenitors (MPP), common lymphoid progenitors (CLP), progenitor (pro-pre-B) and precursor (pre-B) B-lymphocytes—reside within specialized microenvironments, also called niches [1–3]. HSC, MPP, CLP and pre-B cells occupy distinct niches. Niches for HSC are found in perivascular, sinusoidal areas near endothelial cells within bone marrow, while early B-cell progenitors and precursors have been localized in sub-endosteal regions, near the inner bone surface, in the neighborhood of osteoblasts [4, 5]. HSC and their progeny up to pre-BI cells express membrane-bound tyrosine kinase c-kit, which is involved in the regulation of cell proliferation [6]. Kit-ligand (kit-L), also called stem cell factor, is provided in the HSC niche by endothelial cells, in CLP/pro-pre-B- and pre-BI cell niches by mesenchymally-derived osteoblasts, i.e. non-hematopoietic stromal cells. Such stromal cells have been established as cell lines

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in tissue culture [3]. CLP, pro-pre-B- and pre-BI-cells, expressing IL-7 receptors on their surface, require the cytokine IL-7 for survival and, in costimulation with kit-L for proliferation in their niches [7–12]. IL-7 is produced by stromal cells [9]. Furthermore, HSC and their progeny express the chemokine receptor CXCR4 – in fact throughout B-cell development to mature B-cells and plasma cells [5, 13]. However, the capacity to respond by in vitro migration to the ligand of CXCR4, CXCL12, is lost, when pro-pre-B cells develop to pre-BI cells. CXCL12 is abundantly produced by non-hematopoietic stromal cells [14]. In the bone marrow niches CXCR4-expressing HSC and their progeny are thought to be attracted to the CXCL12-producing microenvironments, which provide specific cytokines for proliferation, survival and development, e.g. IL-7 for CLP, pro-pre-B cells and pre-BI cells. When these interactions cease to function for attracting pre-BI cells into IL-7-producing niches, pre-BI cells loose the stimulatory influence of IL-7, are induced to rearrange VDJ-genes within IgH- and IgL-chain loci and develop to pre-BII cells and immature $sIgM^+$ B-cells [7, 8].

It has long been known that mouse precursor B cells of fetal liver, defined as $CD19^+$c-kit^+IL-$7R^+$ D_HJ_H/D_HJ_H-rearranged pre-BI cells, can be cloned and maintained proliferating in vitro at this stage of B-cell differentiation for weeks and months in co-cultures of irradiated messenchymal stromal cells and IL-7 in serum-containing medium [7–10]. Proliferation of pre-BI cells is stimulated through c-kit [11] and the IL-7 receptor [7], while survival is maintained by IL-7 alone [7, 12]. While fetal liver-derived pre-BI cells proliferate for many weeks [8, 10] without changing their state of differentiation, bone marrow-derived pre-BI cells have been found to cease proliferation after two or three weeks on stromal cells and IL-7.

A drawback of this culture system has been, that pre-BI cells might receive a variety of molecularly undefined, hence, experimentally uncontrolled signals from serum and stromal cells. Such signals might hamper an accurate in vitro cell analysis of other, additional co-stimulatory requirements. It has also limited the elucidation of ligand-receptor-dependent signaling pathways and, thus, regulations of gene expression involved in proliferation, survival and differentiation. A previous study reported a serum- and stromal cell-free culture system for pre-BI cells [12]. However, in the initial phase of culture, high cell densities (at 1×10^5/mL) were necessary to establish pre-B cell lines in vitro, cells hardly proliferated during the initial weeks in culture, and it was not possible to clone pre-BI cells, as it was later achieved on stromal cells in serum- and IL-7-containing culture [8].

Here, we report the establishment of a novel culture system for pre-BI cells not only from fetal liver, but also from bone marrow. It allows pre-BI cell cloning at maximum efficiencies and high-rate, long-term proliferation of all pre-BI cells in completely serum-free cultures [15], stimulated by immobilized recombinant kit-L in combination with recombinant, soluble IL-7.

Results and discussion

Fetal liver-derived pre-BI cell culture on immobilized kit-L-Ig

Kit-L is produced in two forms by alternative splicing of the same RNA transcript, allowing the production of a secreted and a membrane-bound form [6, 16]. Both forms are produced by fibroblasts and endothelial cells, and by the stromal cells used in pre-BI cell cultures. We generated kit-L-Ig fusion proteins and coated plastic tissue culture plates at different concentrations with them to mimic membrane-bound forms of kit-L that are normally present on stromal cell surface membranes, and then tested the proliferation of established mouse fetal liver-derived pre-BI cell lines (Fig. 1A). Together with IL-7, plate-bound kit-L-IgFc stimulated long-term proliferation in a dose-dependent manner. Proliferation was maximal, and almost as efficient as on stromal cells, when tissue culture plates were coated at 1 μg/mL. Lower, and also higher concentrations were less stimulatory. The soluble form of kit-L-Ig, or soluble recombinant kit-L did not stimulate pre-BI cell proliferation (Fig. 1A). Microscopic observation showed pre-BI cells on plate-bound kit-L-Ig growing attached to the bottom of the plate, similar to those on stromal cells, while those cultured in the presence of soluble form of kit-L-Ig or recombinant kit-L were floating in medium (Fig. 1B). These results suggest that plate-immobilized kit-L, binding to c-kit receptors on pre-BI cells, crosslinks these receptors for stimulation of proliferation. Too low, or too high densities of kit-L are suboptimal for this stimulation, suggesting, that an optimal pattern of c-kit receptor crosslinking provided optimal stimulation of proliferation.

Next, we replaced serum in the culture medium with purified, Fe^{3+} -complexed transferrin and BSA, coated with defined lipids, that have been found to replace serum for maintaining LPS-stimulated murine B-cells in culture [15]. Previously, different serum-free media had been used to culture pre-B cells [12]. This serum-free medium, IL-7 and an optimal concentration of plate-immobilized kit-L allowed the same number of pre-B cells to proliferate comparably or, two-fold higher, if any, at day 26 of culture (5×10^8 vs. 1×10^9) to conventional culture condition with serum-containing medium on OP9 (Fig. 1C).

In addition, we also added fibronectin and CXCL12. Fibronectin, an extracellular matrix glycoprotein present in serum, is known to play multifunctional roles in cell adhesion, growth and differentiation. We found, that the soluble form of fibronectin together with CXCL12 [17] improved continued pre-BI cell proliferation, again with little significant difference in long-term culture (at day 26 of proliferation 3×10^9 vs. 1×10^9 cells, Fig. 1C). Such pre-BI cells could be maintained at least 3 months without any apparent changes in rates of proliferation, or in their state of differentiation (data not shown). Initial phase of culture on kit-L-Ig in combination with fibronectin and CXCL12 in serum-free condition improved pre-BI cell proliferation two fold higher than that

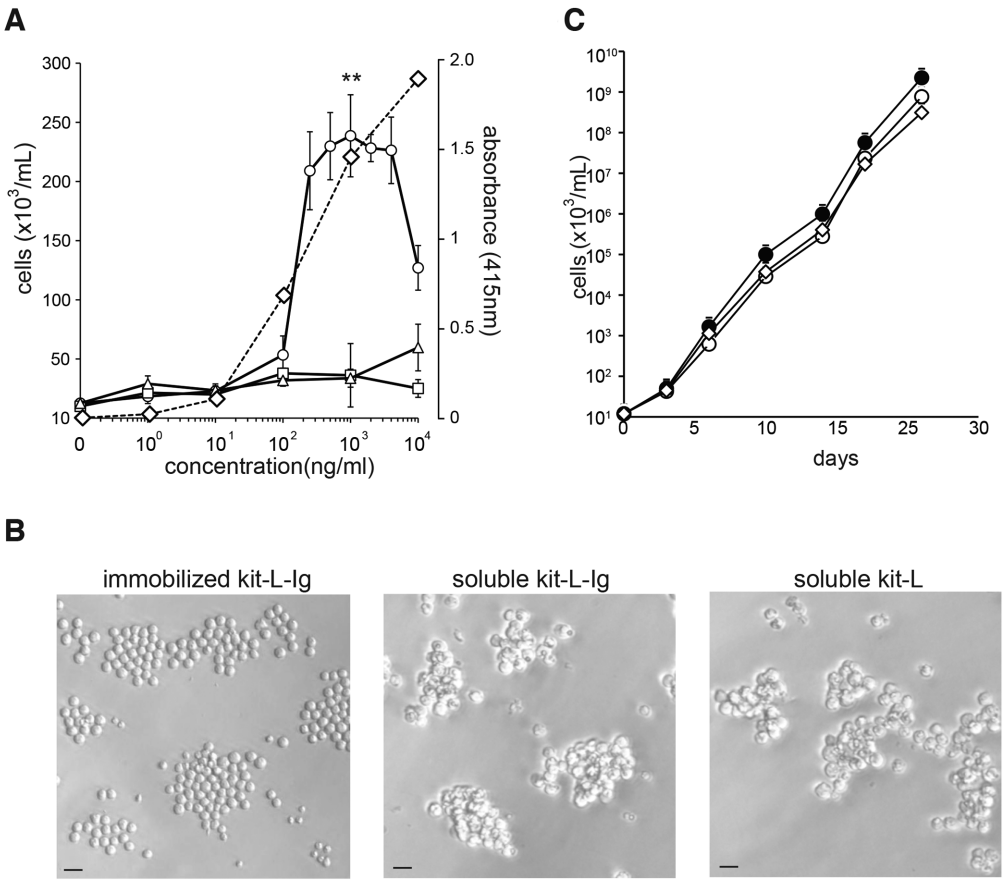

Figure 1. Pre-BI cell cultures on immobilized kit-L-Ig. Fetal liver-derived pre-BI cell lines were cultured in serum-containing medium or serum-free condition medium including IL-7. (A) Cell numbers at day 4 of culture in serum-containing medium on immobilized kit-L-Ig (○), with soluble kit-L-Ig (□) or recombinant kit-L (△) at different concentrations ($n = 4$ each). The number of live cells after culture was determined by flow cytometry. Plate-coated kit-L-Ig, detected by colorimetric assay (dashed line, ◇). (B) Microscopic observation of pre-BI cell lines at day2 cultured on immobilized kit-L-Ig (left), in soluble form of kit-L-Ig (middle) or recombinant kit-L (right). Scale bars indicate 20 μm. Images are from a single experiment representative of at least three independent experiments (C) Kinetics of pre-BI cell proliferation during long-term culture in serum-containing medium with OP9 (◇), or in serum-free condition medium with (●) or without (○) CXCL12 and fibronectin (FN) on immobilized kit-L-Ig. Accumulated cell number counted by flow cytometry at each time point on passage is shown. Data are shown as mean+/– SD and are from a single experiment representative of at least three independent experiments. Statistical analysis was performed by *t*-test; * = $p<0.05$, ** = $p<0.01$. N.S. = not significant.

cultured on kit-L-Ig alone and yielded similar number of pre-BI cells as that on OP9 cells in serum-containing condition (Supporting Information Fig. 1).

Flow cytometric analysis showed that pre-BI cells proliferating on plate-bound kit-L-Ig in serum-free medium were $sIg^-CD19^+IL\text{-}7R^+AA4.1(CD93)^+CD24^+$, but had reduced levels of surface c-kit (CD117) expression, compared with cells proliferating on OP9 stromal cells in serum-containing medium (Supporting Information Fig. 2A). In addition, expression of surface CXCR4 and of surrogate light chain were significantly higher in pre-BI cells maintained on plate-bound kit-L-Ig compared with those on OP9 (Supporting Information Fig. 2A). This elevated CXCR4 expression is possibly due to a loss of receptor down-modulation through CXCL12 produced by OP9, because the addition of CXCL12 in pre-BI cells on plate-bound kit-L-Ig lowered the CXCR4 expression level comparable to that of pre-BI cells maintained on OP9 (Supporting Information Fig. 2B and C). Similarly, higher surface expression of surrogate light chain might be explained by loss of down-modulation through the interaction with the possible ligand on stromal cells, such as heparin sulfates [18, 19].

Serum-free, stromal cell-free cultures of bone marrow-derived pre-BI cells ex vivo

To test if these serum- and stromal cell-free culture conditions could also be used to establish pre-BI cell lines and clones from primary pre-BI cells of fetal liver, or of bone marrow ex vivo, FACS-enriched $CD19^+$c-kit^+ cells from normal mice were cultured in our serum-, stromal cell-free culture conditions. Consistent with cultures of fetal liver-derived pre-BI cell lines (Fig. 1A–C), primary $CD19^+$c-kit^+ cells also proliferated in serum-free medium containing IL-7, CXCL12 and fibronectin on plate-bound kit-L-Ig, with no significant differences to cells cultured on OP9 in serum-contained medium (Fig. 2A).

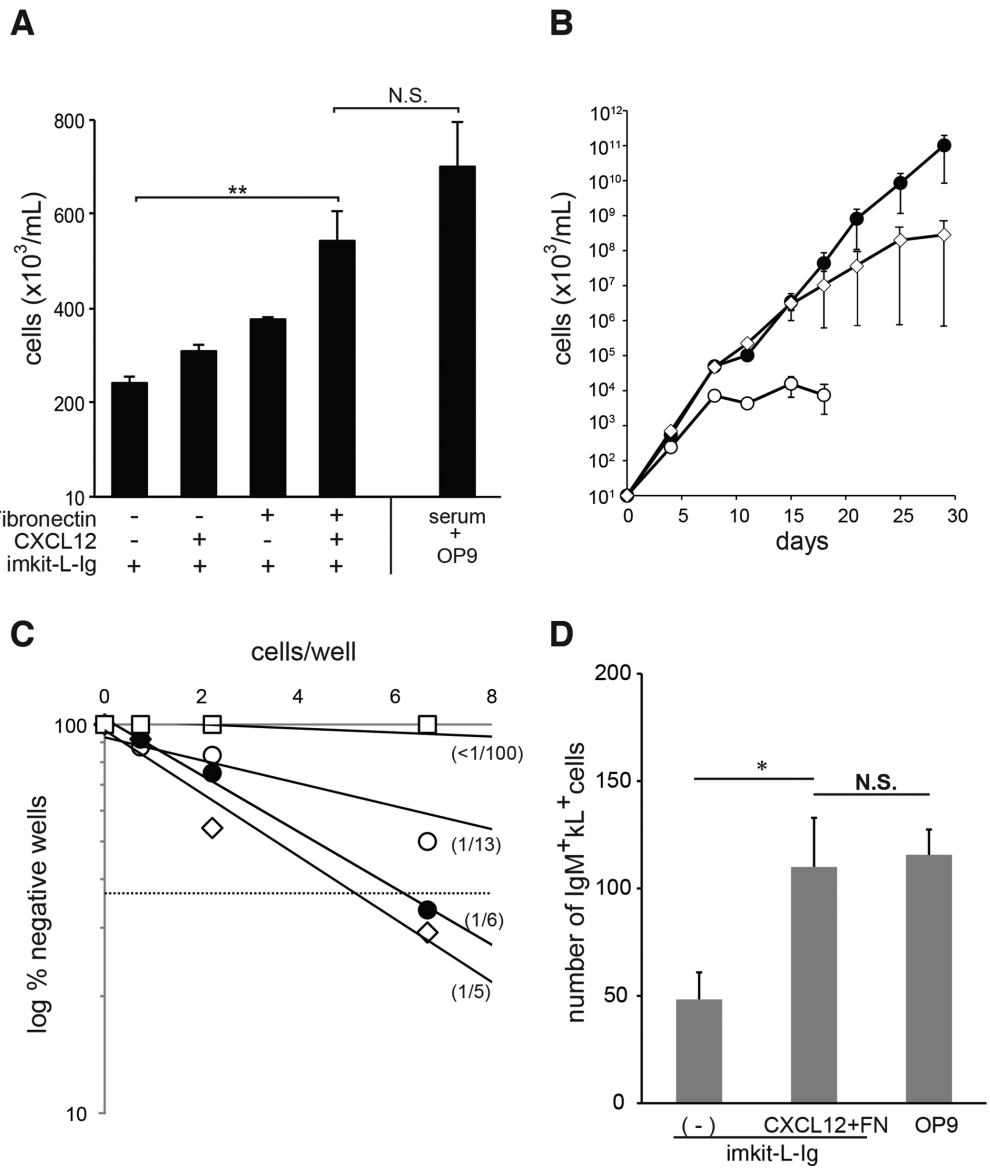

Figure 2. Serum-free, stromal cell-free culture of pre-BI cells ex vivo. FACS-purified bone marrow-derived CD19$^+$ckit$^+$ cells were cultured in culture medium with IL-7 in serum-containing condition on OP9 or in serum-free condition on immobilized kit-L-Ig with or without CXCL12 and/or fibronectin. (A) Cell numbers at day 4 of culture at each culture condition is shown ($n = 4$ each). The number of live cells after culture was determined by flow cytometry. (B) Proliferation kinetics during long-term culture ($n = 3$) and (C) cloning efficiencies at 7 days culture of purified bone marrow-derived CD19$^+$ckit$^+$ cells in serum-containing medium on OP9 (◇), or in serum-free medium with (•) or without (○) CXCL12 and fibronectin on immobilized kit-L-Ig. In (B), accumulated cell number determined by flow cytometry at each time point on passage is shown. In (C), pre-B-cell colonies, consisting of more than 100 cells, were scored by a microscope and the frequency of clonable pre-BI cells was calculated by counting negative wells according to Poisson's distribution. Pre-BI cells cultured in serum-free medium with soluble kit-L-Ig are indicated as open squares (□). (D) The number of IgM$^+$kL$^+$ cells differentiated from pre-BI-cells maintained on indicated culture condition ($n = 4$). Data are shown as mean+/– SD and are from a single experiment representative of at least three independent experiments. A two-tailed paired Student t-test was used for statistical analysis. * = $p < 0.05$, ** = $p < 0.01$. N.S. = not significant.

Notably, cell lines from primary cells of bone marrow could only be established and maintained for long periods of time cultured in serum-free medium with IL-7, CXCL12 and fibronectin on plate-bound kit-L-Ig, but not on OP9 in serum-containing medium (Fig. 2B).

Routinely, CD19$^+$c-kit$^+$CD93$^+$sIg$^-$ pre-BI cells, isolated by FACS ex vivo from fetal liver have been cloned at high efficiencies on OP9 and IL-7 in serum-containing medium culture [8]. Therefore, we tested the efficiency of cloning of such primary pre-BI cells in our serum- and stromal cell-free, plate-immobilized kit-L-Ig-stimulated, IL-7-, fibronectin and CXCL12-containing cultures. Limiting dilution assays revealed that the efficiency of cloning of pre-BI cells on plate-bound kit-L-Ig in the presence of CXCL12 and fibronectin was 1 in 6 cells, and, therefore, almost the same as that

found with cells cultured on OP9 in serum-containing medium, i.e. 1 in 5 cells. The frequency of cloning was two-fold lower in cultures not containing CXCL12 and fibronectin, showing the importance of these molecules. Pre-BI cell cloning efficiency was much lower in cultures containing the soluble form of kit-L-Ig, i.e. 1 in less than 100 cells (Fig. 2C).

Finally, $CD19^+$c-kit^+ pre-BI cells proliferating under the new serum- and stromal cell-free culture conditions were induced to differentiate by the removal from plate-immobilized kit-L-Ig and IL-7 to IgM^+ immature B-cells with frequencies equal to as those previously cultured on OP9 in serum-containing medium (Fig. 2D).

Our results show that a pre-BI cell niche in mouse fetal liver needs no more than IL-7 and immobilized, probably membrane-bound kit-ligand, both provided by the microenvironment, to control proliferative expansions. It is surprising, that these conditions also allow single cell cloning at nearly equally high efficiencies of plating. Hence, stromal cells do not supply any undefined "filler effect" for fetal liver-derived pre-B cells, which thymus cells do for the cloning of mature, LPS-stimulated B-cells [20]. In contrast, our data showed that fibronectin and CXCL12 have a stronger influence to pre-B cells in bone marrow than in fetal liver. Thus, bone marrow-derived pre-B cells need these additional environmental factors to initiate proliferation. This may be a reflection of the capacity of pre-B cells to home to bone marrow niches, and to reside in them, controlled by additional interactions, such as CXCL12-CXCR4, with this microenvironment.

Our new culture system, including CXCL12 and fibronectin, does not allow cloning and proliferation of Pax5-deficient, CLP/MPP-like progenitors [21] with comparable efficiencies (Supporting Information Fig. 3). Additional factors for initiation of growth and for long-term proliferation and survival of CLP, provided in vitro by OP9 stromal cells, and in vivo in CLP niches by the microenvironment, defining the differences in growth requirements between these earlier progenitors and pre-BI cells in early B-cell development might become identifiable with the help of our new culture system.

We expect that a plate-immobilized form of human kit-L in serum-free cultures will provide comparable stimulatory effects for human pre-BI cells. This, in turn, might allow a renewed search for the elusive human, IL-7-like pre-B cell cytokine. In turn, it would set the experimental condition to analyze mutations in human progenitor and pre-B cells, which lead to immunodeficiencies or to malignant transformations in early B-cell development.

In conclusion, we have established a stromal cell-free, serum-free culture system for primary $CD19^+$c-kit^+ cells as well as pre-BI cell lines, which is functionally equivalent to previously used cultures on stromal cells. These conditions also allow us to establish pre-BI cell lines and clones from bone marrow, which has previously not been possible. Hence, fetal liver and bone marrow appear to have functionally distinct niches, in which they proliferate. Besides, this simple culture platform will be of technical advantage to allow drug-based selection, free of drug-resistant stromal cells for the generation of gene-modified pre-BI cells, as well as useful for studies of intracellular signaling pathways, controls of gene expression and functional responses of single pre-BI cells in defined, ligand-controlled ways.

Materials and methods

Animals

C57BL/6 mice (Charles River, Germany) were maintained under specific pathogen-free (SPF) conditions. All experiments were performed according to German guidelines for animal experiments.

Vector construction, production and purification of kit-L-Ig

We designed two gene fragments of double strand DNA nucleotide sequences generated by artificial gene synthesis (Life technologies), consisting of the mouse immunoglobulin kappa chain signal peptide (METDTLLLWVLLLWVPGSTGD) followed by the extracellular domain of mouse kit-L (1-164 a.a of mature peptide from NCBI Reference Sequence: NP_038626.1), and the constant region of mutant human IgG1 (non-lytic form, 6–232 a.a from GenBank: AEV43323.1, where Leu at position 20 was mutated to Glu, and Glu, Lys, Lys at position 103, 105, 107, respectively, was mutated to Ala) [22] with 3xGGGGS linker sequences at the N-terminus. These fragments were inserted into the cloning site of a mammalian expression vector pCAGGS [23] by In-fusion cloning (Clonetech) to generate kit-L-Ig expression vector. The plasmid (30 μg) was transfected into 293T cells by 90 μg of PEI-MAX (Polyscience) for the transient overexpression to produce kit-L-Ig fusion proteins in the culture supernatant, followed by column purification with protein A. The proteins after elution with 0.1M glycine buffer (pH 2.7) were immediately neutralized with 1M Tris-EDTA buffer (pH 9.0), and a part of them was subjected to SDS-PAGE followed by the staining with Coomasie Brilliant Blue G-250 (Supporting Information Fig. 4). After the confirmation of purity (>90%), eluted fractions were dialyzed with PBS and sterilized by 0.22 μm filter. Protein concentration was determined by Bradford assay (Bio-rad) using NanoDrop 1000 (Thermo Scientic).

The measurement of plate-coated kit-L-Ig

Different concentrations of kit-L-Ig in PBS 50 μL were coated on 96-well plate for 2 h at RT. After washing 3 times with 200 μL of PBS/0.05%tween-20, 100 μL of PBS/0.1%BSA solution was added and incubated for 1o/n at 4ºC. After removal of blocking

buffer, plates were incubated with 100 μL of HRP-conjugated goat anti-human IgG (Santacruz, sc-2453) at 1 μg/mL for 1 h at RT. Following extensive wash 5 times with PBS/0.05%tween-20, 100 μL of 1xTMB solution (ebioscience) was added and the absorbance at 450 nm was measured by microplate reader Model 680XR (Bio-rad).

Cytokines and medium

Culture medium was prepared in Iscove's Modified Dulbecco's Medium (IMDM) supplemented with 0.036 mM $NaHCO_3$/50 μM β-mercaptoethanol/0.03% primatone/1x kanamycin/1x non-essential amino acid (Gibco) including 1 ng/mL IL-7 (Peprotech) together with 2% fetal bovine serum (FBS) or 1 mg/mL of de-lipified BSA (Sigma), 10 μg/mL of human transferrin (Sigma) and 0.005%Fatty-acid Supplement (Sigma) (designated as serum-free conditioned medium). In some experiments, 100 ng/mL of CXCL12α (Peprotech) and/or 10 μg/mL Fibronectin (Sigma) were added in culture.

Cell culture

Fetal liver-derived pre-BI cell lines or bone marrow-derived $CD19^+ckit^+$ cells isolated by FACS from 6–8wks-old C57BL/6 mice, or Pax5-deficient proB-cell lines were plated at 1×10^4/mL in 200 μL of serum-containing medium or serum-free condition medium, including 1 ng/mL IL-7 on 96-well flat-bottom plates. OP9 cells were pre-coated at 1×10^4 cells/cm^2 one day before 30-Gy irradiation. Kit-L-Ig proteins were pre-coated at different concentration ranging from 1 ng/mL to 10 μg/mL by tenfold serial dilution by the incubation at room temperature for at least 2 h, followed by wash 3 times with PBS before cell culture. Kit-L-Ig or recombinant kit-L (Peprotech, #250-03) were also added in culture as soluble form at the same concentration as that used for immobilization of kit-L-Ig. Cells were passaged every 3 or 4 days to another wells freshly pre-coated with OP9 cells or kit-L-Ig, and cell proliferation was evaluated at each time point after culture by counting the number of live cells determined as negative for propidium iodide by LSR II (BD) analyzer. Data analysis was performed with FlowJo (Treestar).

Limiting dilution assay

FACS-sorted $CD19^+ckit^+$ cells were plated at 4 different number of cells by threefold serial dilution from 20 cells to 0.7 cells in 24 wells each on 96-well plates coated with irradiated OP9 cells in serum-containing medium or with 1μg/mL of kit-L-Ig in serum-free condition medium with or without CXCL12 and fibronectin. After 7 days of culture, pre-B cell colonies, consisting of more than 100 cells, was scored by a microscope and the frequency of clonable pre-BI cells was calculated by counting negative wells according to Poisson's distribution.

Statistics

Statistical analyses were performed by using a two-tailed paired Student *t* test. *P*-value of <0.05 was considered statistically significant.

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Conflict of interest: The authors declare no commercial or financial conflict of interest.

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Abbreviations: **BSA**: bovine serum albumin · **CLP**: common lymphoid progenitors · **FN**: fibronectin · **HSCs**: hematopoietic stem cells · **Ig**: immunoglobulin · **IMDM**: Iscove's modified Dulbecco's medium · **Kit-L**: kit-ligand · **LPS**: lipopolysaccharide · **MPP**: multipotent progenitors · **pre-B**: precursor B cells · **pro-pre-B**: progenitor of precursor B cells

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